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BIOTECHNOLOGY and BIOENGINEERING

# Impedance Spectroscopy as a Tool for Non-Intrusive Detection of Extracellular Mediators in Microbial Fuel Cells

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Received 5 May 2009; revision received 29 June 2009; accepted 2 July 2009

Published online 7 July 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22469

**ABSTRACT:** Endogenously produced, diffusible redox mediators can act as electron shuttles for bacterial respiration. Accordingly, the mediators also serve a critical role in microbial fuel cells (MFCs), as they assist extracellular electron transfer from the bacteria to the anode serving as the intermediate electron sink. Electrochemical impedance spectroscopy (EIS) may be a valuable tool for evaluating the role of mediators in an operating MFC. EIS offers distinct advantages over some conventional analytical methods for the investigation of MFC systems because EIS can elucidate the electrochemical properties of various charge transfer processes in the bio-energetic pathway. Preliminary investigations of *Shewanella oneidensis* DSP10-based MFCs revealed that even low quantities of extracellular mediators significantly influence the impedance behavior of MFCs. EIS results also suggested that for the model MFC studied, electron transfer from the mediator to the anode may be up to 15 times faster than the electron transfer from bacteria to the mediator. When a simple carbonate membrane separated the anode and cathode chambers, the extracellular mediators were also detected at the cathode, indicating diffusion from the anode under open circuit conditions. The findings demonstrated that EIS can be used as a tool to indicate presence of extracellular redox mediators produced by microorganisms and their participation in extracellular electron shuttling.

Biotechnol. Bioeng. 2009;104: 882–891.



**KEYWORDS:** microbial fuel cells; electrochemical impedance spectroscopy; redox mediators; *Shewanella oneidensis*; riboflavin

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## Introduction

Mediated electron transfer using endogenously produced, redox-active soluble molecules is one among the several mechanisms that bacteria use for anaerobic respiration. The molecules effectively shuttle electrons from the respiratory cascade to alternate electron acceptors in the absence of oxygen. In recent years, particular interest has focused upon bacteria that use insoluble metal oxides as electron acceptors. The phenomenon influences geochemical processes and may be exploited in treatment processes for environmental bioremediation (Lovley, 2006). Similarly, extracellular electron transfer is the intrinsic component for electricity generation in microbial fuel cells. In the microbial fuel cell (MFC), the anode serves as the electron acceptor for bacteria present in the system. Defining the mechanism and predicting the conditions that allow certain microorganisms to efficiently deliver electrons to an insoluble electron acceptor is a key to understanding the biology of MFC and to using the process for practical means.

*Shewanella oneidensis* is a widely studied microbe for MFCs and is known to synthesize a suite of potential endogenous redox mediators including menaquinones, flavins and ubiquinones in order to facilitate extracellular electron transfer to insoluble electron acceptors (Lies et al., 2005; Marsili et al., 2008; Myers and Myers, 2004; Newman and Kolter, 2000; Rosso et al., 2003; vonCanstein et al., 2008; Ward et al., 2004). In an earlier study (Biffinger et al., 2008) that examined a *Shewanella*-based MFC, endogenously produced flavins were detected and identified using HPLC analysis. Other work described by vonCanstein et al. (2008) estimated that *Shewanella oneidensis* MR-1 secretes FMN, FAD and riboflavin in the concentration range of 100–500 nM after 1 week of operation. Similarly, Marsili et al. (2008) reported that strain MR-1 accumulated 250–500 nM of riboflavin after 4 days in a test MFC. Other microorganisms such as *Geothrix* (Nevin and Lovley, 2002),

# Report Documentation Page

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| **2009** | | **00-00-2009 to 00-00-2009** |

| 4. TITLE AND SUBTITLE | 5a. CONTRACT NUMBER |
|---|---|
| **Impedance Spectroscopy as a Tool for Non-Intrusive Detection of Extracellular Mediators in Microbial Fuel Cells** | 5b. GRANT NUMBER |
| | 5c. PROGRAM ELEMENT NUMBER |
| 6. AUTHOR(S) | 5d. PROJECT NUMBER |
| | 5e. TASK NUMBER |
| | 5f. WORK UNIT NUMBER |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Air Force Research Laboratory,Tyndall AFB,FL,32403** | 8. PERFORMING ORGANIZATION REPORT NUMBER |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | 10. SPONSOR/MONITOR'S ACRONYM(S) |
| | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) |

12. DISTRIBUTION/AVAILABILITY STATEMENT
**Approved for public release; distribution unlimited**

13. SUPPLEMENTARY NOTES
**Biotechnology and Bioengineering, Vol. 104, No. 5, December 1, 2009**

14. ABSTRACT

15. SUBJECT TERMS

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT **unclassified** | b. ABSTRACT **unclassified** | c. THIS PAGE **unclassified** | **Same as Report (SAR)** | **11** | |

*Pseudomonas* (Hernandez et al., 2004; Rabaey et al., 2005), *Lactobacillus* and *Enterococcus* (Rabaey et al., 2004) species also appear to secrete soluble electrochemically active compounds in order to mediate extracellular electron transfer.

A variety of biochemical and spectroscopic methods are available to detect and analyze extracellular mediators from microbial fuel cell media. Approaches include quantitative methods (vonCanstein et al., 2008) such as azo-dye based redox mediator assays, UV-vis spectroscopy, LC-MS and HPLC, as well as qualitative electrochemical techniques (Marsili et al., 2008) such as cyclic voltammetry (CV) and differential pulse voltammetry (DPV). For example, redox assays would provide information about the concentration (mol/L) of the relevant compound present in spent anolyte, while UV-vis, LC, and HPLC techniques help identify a specific redox compound and subsequently quantify the concentration of that compound upon the availability of suitable parameters. Electrochemical methods such as CV and DPV (Marsili et al., 2008) have been used to detect the presence of redox compounds in spent anolyte solution based on their reversible electrochemical activity (in the form of a current peak) near the predicted redox potential for that compound. Each of these techniques offer unique advantages, however, all the above techniques are intrusive and require that the MFC is accessed or even disassembled, potentially leading to a discontinuity in the testing. Moreover, these techniques do not provide direct online measurements within the MFC, therefore the mediator influence on the electrochemical behavior of an operating MFC cannot be determined directly. CV and DPV can provide insight to the bioelectrochemistry of an MFC and may be adapted to an in situ analytical method. However, both techniques transiently change the voltage (hence the steady operation) of the system during measurement and in turn could influence the bacteria or MFC behaviors. Possible peturbations include, rate of electrochemical reaction(s), rate of generation and consumption of extracellular mediators, and changed over-potentials on either half-cell electrode. EIS is a steady state electrochemical technique in that the measurements are made without altering the current–voltage properties of the microbial fuel cell, thereby avoiding any deviation from its normal operation.

He and Mansfeld (2009) suggested in their recent review that under properly designed experimental conditions, EIS may be used to detect mediators in MFCs. In the present work, electrochemical impedance spectroscopy (EIS) was used as a non-intrusive tool to identify and elucidate the electrochemical properties of redox mediators produced by microbes. EIS enabled the study of charge transfer behavior of mediators and their impact on the MFC impedance without a need to interrupt the MFC operation. Furthermore it can be used to examine the relative significance of mediator reaction(s), among the many charge transfer processes that occur in the bio-energetic pathway. Although impedance spectroscopy had been used previously to study the impedance behavior of electrodes and biofilm in MFCs (He et al., 2006, 2008; Manohar et al., 2008; Ramasamy et al., 2007, 2008a,b,c), this is the first known attempt to use the technique to detect and determine the properties of endogenously secreted extracellular mediators in an active MFC.

A simplified electron transfer pathway between the substrate and terminal electron acceptor involving electron shuttles or mediators is schematically shown in Figure 1. *Shewanella oneidensis* DSP10, which had been confirmed by several groups to secrete extracellular mediators, was chosen as the anode biocatalyst in this study. At the anode, *Shewanella* oxidizes lactate to generate electrons, which are transferred to the endogenously produced electron shuttle (ES). The impedance to this charge transfer reaction is given the notation $R_A$. The electron shuttle then transfers electrons to the graphite felt anode upon overcoming the impedance of a second charge transfer reaction, $R_{ES}$. The electrons are then transferred via the external circuit to the cathode where it reduces the terminal electron acceptor upon overcoming the charge transfer impedance for the cathodic reaction, $R_C$. The hypothetical model in Figure 1 assumes that mediators are the sole carriers of electrons between the bacteria and anode. The assumption is appropriate due to an intrinsic

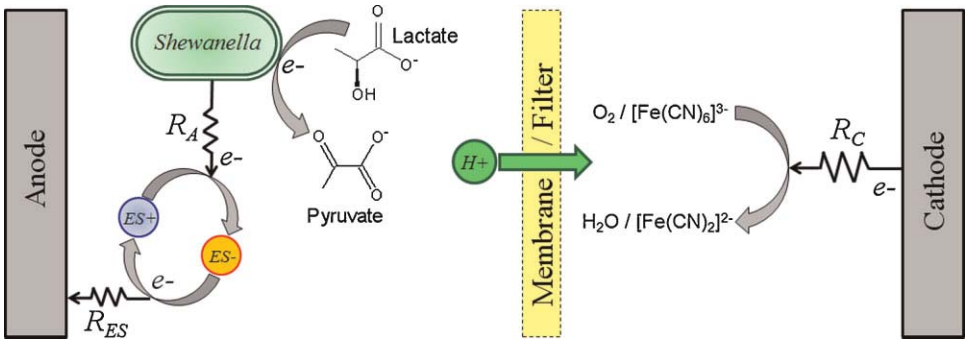


**Figure 1.** Sequence of MFC electrochemical reactions and their corresponding charge transfer resistances: $R_A$, electron transfer from substrate; $R_{ES}$, electron transfer from electron shuttle; and $R_C$, electron transfer to oxidant reduction at the cathode. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

aspect of the EIS technique and from quantitative measurements. Other potential extracellular electron transfer mechanisms involving outer membrane cytochromes and/or bacterial nanowires would be embedded in the $R_A$ impedance and can be disregarded in the present analysis (Ramasamy et al., 2008b). In addition, the anode open circuit potentials in the operating MFCs were consistently below −0.2 V versus standard hydrogen electrode (SHE), suggesting that electron transfer through outer membrane *cytochrome-c* (redox potential >0.0 V vs. SHE) (Logan and Regan, 2006) is not a major mechanism in the MFCs analyzed.

Previous work showed that DSP10 strain secretes mediators during cultivation at low oxygen concentrations (Biffinger et al., 2008). The objective of the present study is to distinguish the impedance contributions of substrate oxidation ($R_A$) and mediator redox reactions ($R_{ES}$) at the anode, as well as to determine the relative significance of mediator charge transfer process, without the need to interrupt MFC operation or involve ex situ sampling.

## Materials and Methods

### Cell Design and Configuration

Three distinct MFC configurations were used in order to examine EIS response due to mediator presence. The first approach intended to confirm EIS responsiveness by supplementing the MFC with known concentration and type of soluble mediator. This reactor included an air-breathing cathode (ETekTM ELAT® 120EW GDE, BASF, Florham Park, NJ) that obviated reliance on oxygen dissolution in culture medium. Other components and electrolytes in MFC were identical to the two chambered MFCs described below.

Two chamber MFCs with separate anode and cathode chambers were used for other experiments in this study. The terminal electron acceptor at the cathode was either ferricyanide or oxygen. For oxygen based MFCs, the culture medium served as the electrolyte in both chambers and a polycarbonate filter (0.4 μm pore diameter) (Millipore, Billerica, MA) was used as the separator between the anode and cathode chambers. The polycarbonate membrane allows free movement of ions between the electrodes and acts essentially as a bacterial filter. Comparably porous, traditional separators used in other electrochemical systems do not work well in the physiological pH range and the polycarbonate material is a low cost alternative to PEM fuel sell separators such as Nafion (DuPont, Inc., Wilmington, DE). Graphite felt (0.47 $m^2$/g) was used for the cathode, (Electrosynthesis, Lancaster, NY). In the ferricyanide-based MFC, the cathode and anode chambers were separated with a cation exchange membrane (CEM) (Ultrex, Membranes International, Glen Rock, NJ). Porous graphite felt (~20 $cm^2$ area) served as the anode in both MFCs. The reactors were operated in batch mode. External aeration was deliberately eliminated in both MFCs to evaluate the performance under passive aeration. Therefore, the oxygen based MFC relied solely on the dissolved oxygen in the electrolyte medium for the cathodic electron sink.

### Growth Medium and Bacterial Cultivation

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO). *Shewanella oneidensis* DSP10 was typically cultivated in a 250 mL baffled shaker flask using 50 mL Luria-Bertani (LB) broth. Rifampicin (5 mg/L) was used as an antibiotic to maintain selection for strain DSP10. The culture was incubated at 25°C using modest agitation (100 rpm) on a shaking platform. The MFC trials were initiated with 5 mL of overnight cultures (optical density ~2.0 at 600 nm) and then transferred into the anode chamber of the MFC containing 75 mL of the chemically defined media used by Myers and Nealson (1988) that had been supplemented with 20 mM lactate (LSMM). The anolyte samples were analyzed regularly using HPLC to ensure ample lactate availability (>5 mM). Serial dilutions of the anolyte were frequently cultivated on nonselective LB agar plates in order to confirm that an axenic culture was maintained. Mediator supplements were added to the MFCs only when used in the validation study. For the MFC used in validation studies the defined media was modified according to Gorby et al. (2006) and supplemented with specific concentrations of riboflavin.

### Electrochemical Testing

Voltage data were acquired continuously using DAQ/54 modules (I/O Tech, Cleveland, OH) across a known resistor. The MFCs were operated under a load of 330 Ω for more than 500 h to ensure complete biofilm growth on the anode. Electrochemical impedance spectroscopy (EIS) tests were performed using a Versastat FRA analyzer (Princeton Applied Research, Oak Ridge, TN). An alternating current (*ac*) signal with amplitude of ±10 mV, between frequencies 10 kHz and 10 mHz was used for all EIS experiments. The measurements were made at open circuit conditions on both the anode and cathode using a saturated Ag/AgCl (Pine Instruments, Grove City, PA) reference electrode placed in close proximity to the anode. Individual resistances were obtained by equivalent circuit fitting analysis (maximum error tolerance of 2%) of the resulting data using the ZSimpWin software integrated with the Versastat instrument.

## Results and Discussion

### Influence of Riboflavin on the EIS Response

The effect of extracellular mediators on the EIS response was studied by intentionally altering the anolyte composition in

a lactate-fed air breathing MFC. EIS measurements were performed on this MFC at open circuit both before and after supplementing with 5 μM riboflavin to act as the redox mediator. The supplemented mediator concentration is 10- to 20-fold higher than the reported concentration of endogenously produced mediators in *S. oneidensis* (Marsili et al., 2008; vonCanstein et al., 2008). The intentionally high concentration of riboflavin supplement amplified the EIS response and avoided any limitations related to riboflavin. Figure 2 shows the anode Nyquist plots before and after supplementing the anode with riboflavin. Two distinct Nyquist arcs were observed corresponding to redox processes in the medium and low frequency domains (Fig. 2). Generally the bio-electrochemical substrate oxidation processes are slow, offer high impedance and are exhibited in the mid-to-low frequency domains depending on the type of MFC (Ramasamy et al., 2008b). The Nyquist arc within the low frequency domain in Figure 2 is likely due to the charge transfer impedance (CTI) for substrate oxidation, $R_A$ (see Fig. 1). The Nyquist arc observed in medium frequency region in Figure 2 has not been observed in previous EIS examinations on mixed culture MFC systems (He et al., 2008; Ramasamy et al., 2007, 2008a,b). We conclude that this can be attributed to the CTI of the electron shuttle redox processes at the anode, $R_{ES}$ (see Fig. 1). The designation of $R_{ES}$ and $R_A$ to the medium and low frequency redox processes is consistent with the EIS response following riboflavin addition. Supplementing the MFC with 5 μM riboflavin decreases the CTI for substrate oxidation ($R_A$), indicated by the reduction in the magnitude of low frequency Nyquist arc, enhancing the kinetics of electron transfer from the substrate to anode. The observations are consistent with a charge transfer process in which substrate oxidation ($R_A$) acts as the rate limiting step, since it is significantly slower than the mediator charge transfer process ($R_{ES}$) and ferricyanide or oxygen reduction steps ($R_c$) which are schematically shown in Figure 1.

## EIS Response of Active, Two-Chamber MFCs

EIS measurements of the ferricyanide and oxygen based MFCs were made under open circuit conditions after extended system acclimation (>500 h under load) with the aim to detect the endogenously produced mediators in non-polarized MFCs. Figure 3 shows the anode and cathode Nyquist plots for both oxygen as well as ferricyanide based MFCs. When ferricyanide is the terminal electron acceptor, the shapes of anode (Fig. 3a) and cathode (Fig. 3b) Nyquist plots were distinct. Since ferricyanide reduction is a fast process, there was little impedance to the charge transfer ($R_C$) and hence did not extend into the low frequency region of the Nyquist plots. By comparison, the bio-electrochemical oxidative processes at the anode are kinetically slow. Accordingly, the Nyquist plots showed a high charge transfer resistance ($R_A$), and thereby extended into the low frequency region, results consistent with previous observations (He et al., 2008; Manohar et al., 2008; Ramasamy et al., 2007, 2008a,b,c). On the other hand, when oxygen was used as the terminal electron acceptor, the cathode plots (Fig. 3b) show the onset of a second Nyquist arc at low frequencies. The second arc corresponds to the

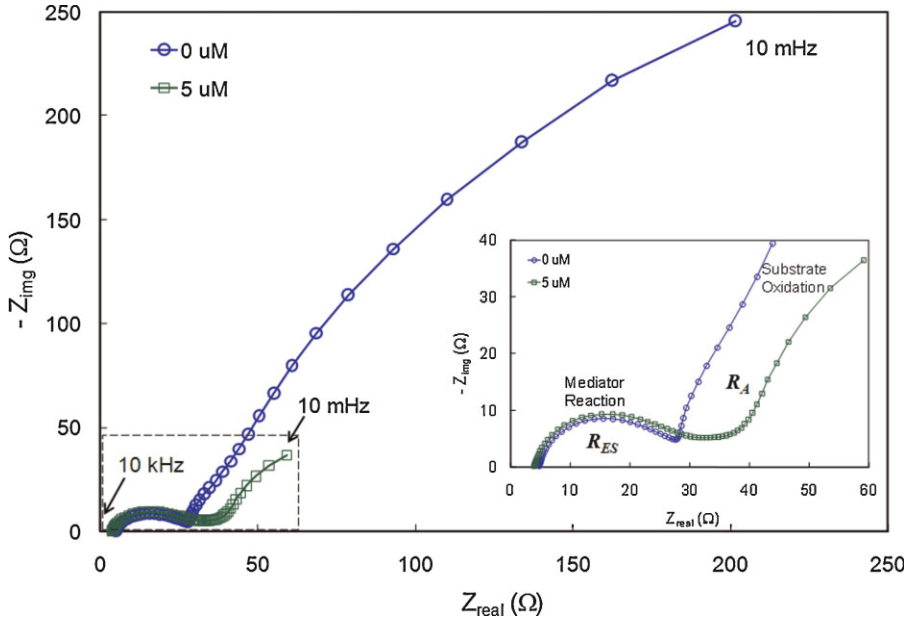


**Figure 2.** Nyquist plots of the anode between frequencies 10 kHz and 10 mHz before and after the addition of 5 μM riboflavin to the anolyte. Inset graph show the expanded view of the boxed region. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

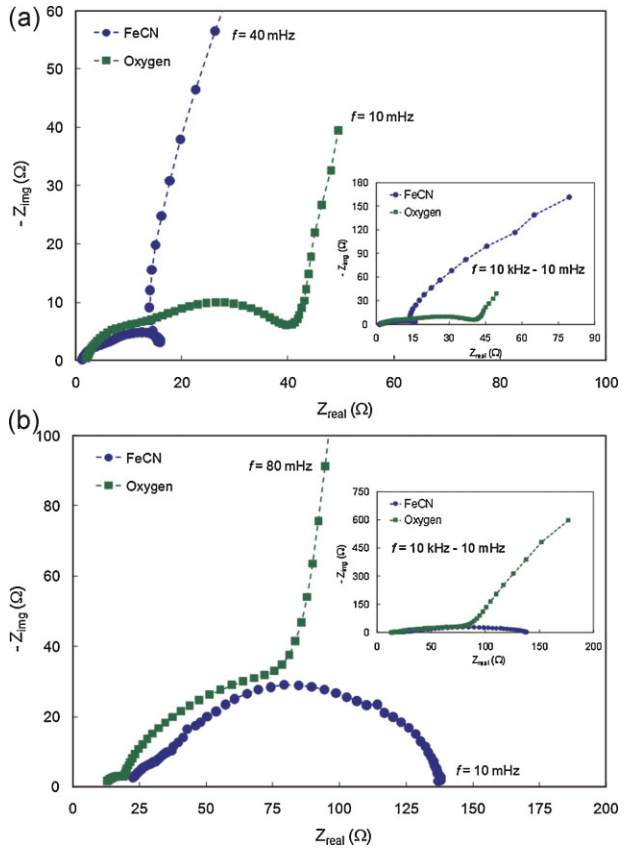


**Figure 3.** Nyquist plots for ferricyanide (circles) and oxygen (squares) based MFCs for: (**a**) anode and (**b**) cathode. Inset graphs show the Nyquist plots for the entire frequency range (10 kHz to 10 mHz). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

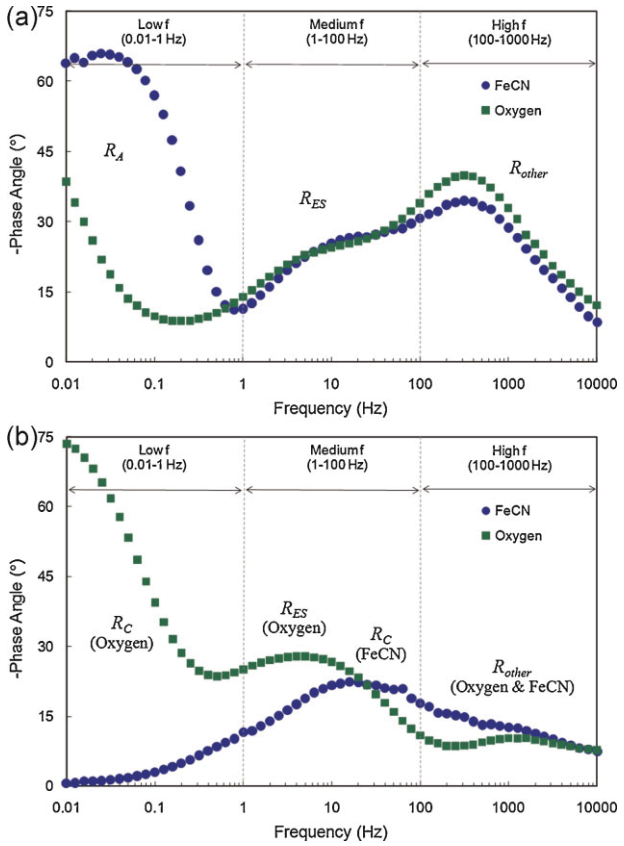


**Figure 4.** Bode phase angle plots for ferricyanide (circles) and oxygen (squares) based MFCs for: (**a**) anode and (**b**) cathode. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

higher impedance for oxygen reduction on graphite felt compared to the reduction of ferricyanide (Ramasamy et al., 2008a,b,c). Although the catalytic limitations strongly affect the observed behavior, the measured impedance was likely also influenced by charge transfer and mass transfer effects. Due to differences in biocatalyst properties in the reactors (cultivation history, extent and variation of the biofilm), the anode impedance behavior was not identical for the separate oxygen- and ferricyanide-based model MFCs used in this study. Nonetheless at any given *ac* frequency, the overall anode impedance (|Z|) for both MFCs were comparable.

## Detection of Endogenously Synthesized Mediators

The occurrence of more than one electrochemical reaction was identified in the high-medium frequency region for both anode and cathode. The change in the Nyquist plots is a subtle deflection in the high frequency arc. The detection of the phenomenon is prominent in the Bode phase angle plots shown in Figure 4, which distinguishes electrochemical reactions based on their time constants for *ac* response. The frequency at which the phase angle plots reach the maxima is determined by the charge transfer resistance, that is, high charge transfer resistance will decrease the frequency of phase angle maxima.

### *Anode*

The anodes in both ferricyanide and oxygen based MFCs (Fig. 4a) exhibited three time constants (represented by the bell shaped curve in phase angle plots) one each in the low, medium and high frequency regions. Each of these time constants correspond to a faradaic process, in this case a charge transfer. As discussed above, the anodic process at low frequencies in Figure 4a is attributed to the charge transfer impedance (CTI) for substrate oxidation, $R_A$ (Fig. 1). It is unlikely that any biochemically derived redox compounds, that is, synthesized mediators, yield a *complete* faradaic response to an *ac* signal faster than 100 Hz. Hence, the reaction in the high frequency region depicts a fast electrochemical process such as oxidation of soluble metal ions in the growth medium. Although the process denoted by $R_{other}$ in Figure 4a, is not included in our hypothetical electron transfer sequence (Fig. 1), it does not come at a surprise as the culture media (Myers and Nealson, 1988)

used in this study contained a variety of metallic salts in micromolar concentrations that can be readily oxidized or reduced in response to *ac* signal, even at open circuit conditions. Results from EIS measurements of bare graphite felt in a separate cell with the same base medium as electrolyte confirmed that the high frequency processes corresponding to $R_{other}$ occur in the absence of bacteria or substrate and hence are not relevant to the bio-electrochemical reactions (See Appendix Fig. A1). It is expected that when MFCs are polarized under load, the effects of $R_{other}$ would be minimized.

The critical observation in our study is the detection of a new process in the medium frequency region (Figs. 2 and 4a). This response has not been reported before for MFC systems and we attribute the mid-frequency process to the charge transfer between the endogenously synthesized mediator and the electrode ($R_{ES}$ in Fig. 1). Even though the mediator redox processes may possess high electron transfer rate constants, the charge transfer resistance offered by these redox processes was significant, likely due to low mediator concentrations in the electrolyte. The results demonstrate that EIS responds to the presence of endogenously produced mediators without the interference of response from redox processes for substrate oxidation.

### *Cathode*

The physiological role of the mediator is related to bacterial respiration, accordingly, their influence should manifest in the anodic half-cell. The design of the MFC reactor, however, will influence the mediator distribution within the system. The distribution may lead to experimental artifacts within the cathode. In the ferricyanide-based two-chamber MFC, the cationic exchange membrane (CEM) effectively separated the anolyte and catholyte to prevent diffusion of mediator to the cathode. But the filter membrane used as a separator in the oxygen-based MFC will not prevent the transport of small molecules and mediator transport from anode to cathode is likely. After prolonged batch mode operation, the mediators produced by the microbes in the anode half cell may be dispersed throughout the electrolyte. In the cathode half-cell, the mediators can likewise act as electron shuttles and partake in the oxygen reduction reaction. The extent of the mediator effect on the EIS response would depend on the concentration, electrochemical properties, and importantly, the cathode potential. It is critical to note that mediators will maintain their equilibrium concentrations under open circuit conditions even while responding to the *ac* stimulus of the EIS. Accordingly, studying the influence of endogenous mediators on the cathode is relevant in the MFC, although that is not the intended role of the redox molecules from the biocatalyst's perspective.

The Bode phase angle plots for ferricyanide and oxygen based cathodes shown in Figure 4b clearly distinguish the mediator effects on these cathodes. The ferricyanide based cathode was essentially separate from the anolyte due to the CEM and the catholyte did not show any evidence of endogenous mediators. This was confirmed by the presence of only two time constants in the Bode plots for the ferricyanide cathode (Fig. 4b), one each in the high and medium frequency regions. The high frequency processes was attributed to metal salts in the electrolyte and the second event, ferricyanide reduction. Since kinetics of ferricyanide reduction is fast relative to oxygen reduction, the charge transfer process corresponding to this reduction occurs in the medium frequency range ($R_C$ for FeCN in Fig. 4b). A distinct time constant for the mediator redox process was not observed in the ferricyanide catholyte and there was no apparent electrochemical process in the low frequency region. The results concur with the Nyquist plots (Fig. 3b), where ferricyanide did not show a capacitive behavior in the low frequency domain.

The measurements from the oxygen based cathode exhibited three time constants in Figure 4b, one in each frequency domain similar to the anode. The result is consistent with the permeable separator allowing the transport of mediators from anode to cathode. The polycarbonate filter's average pore size of 0.4 μm will not affect mediator transport throughout the MFC. Moreover, since there is no anodic over-potential under open circuit conditions, the mediators could readily interact with the cathode. Therefore the time constant in the medium frequency domain (Fig. 4b) can be attributed to the mediator redox process at the oxygen based cathode, with a charge transfer impedance $R_{ES}$. As above, the redox processes at high frequencies can be attributed to metallic compounds in the medium ($R_{other}$).

## Resistances of Individual Redox Processes

The EIS data were analyzed using an equivalent circuit fitting method in order to extract the charge transfer impedance for each participating electrochemical process detectable in the phase angle plots. It is obvious from the Nyquist (Fig. 3) and Bode phase angle (Fig. 4) plots that dissimilar cathodic processes occur in ferricyanide and oxygen based MFCs. Hence using the same equivalent circuit for both cathodes would lead to incorrect description of the actual processes. Therefore more descriptive equivalent circuits customized for each electrode were used for fitting the data to describe parameters specific to that MFC. Figure 5 shows the equivalent electrical circuits used for fitting the anode and cathode Nyquist data. The circuit consists of an electrolyte ohmic resistance ($R_{ohm}$), and three sets of parallel resistor—capacitor elements connected in series, one for each charge transfer process discussed above. The circuit model for the anode (Fig. 5a) captures the electron transfer mechanisms involving $R_A$, $R_{ES}$, and $R_{other}$. A similar equivalent circuit was used to describe the cathodic processes at the oxygen electrode (Fig. 5b) with the only difference being the direction of electron transport as depicted in Figure 1. Due to the absence of the mediator

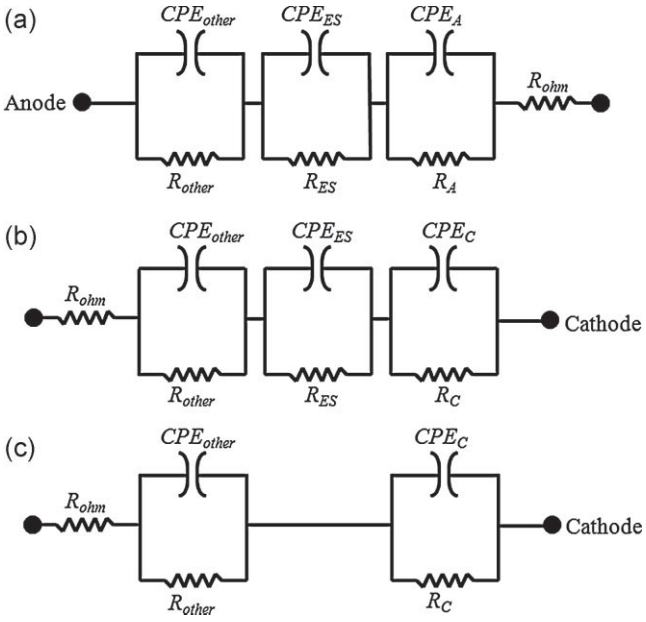


**Figure 5.** Equivalent electrical circuits used to fit the EIS data. **a**: Anodes; (**b**) oxygen based cathode; and (**c**) ferricyanide based cathode.

redox process at the ferricyanide cathode, the data were fitted with two sets of resistor-capacitor elements (Fig. 5c). Due to the non-ideal capacitive behavior of the porous graphite felt in all cases, a constant phase element (CPE) was used in place of a true capacitor. If direct transfer of electrons through the outer membrane cytochromes and/or microbial nanowires is practiced by the organisms in the present MFC, it is unlikely to possess unique capacitive behavior (Ramasamy et al., 2008b) and will not be observed in the impedance plots.

The values for ohmic ($R_{ohm}$) and charge transfer impedances ($R_A$, $R_C$, $R_{ES}$, and $R_{other}$ for the anode and cathode) obtained by equivalent circuit fitting are reported in Tables I and II respectively. Note that the resistances are reported as absolute values and were not normalized. As expected, the ohmic resistances on the anode were lower (Table I), likely due to the close proximity of the reference electrode to the anode. It is interesting to note that for both ferricyanide and oxygen based MFCs, the CTI of substrate oxidation reaction ($R_A$) is about an order of magnitude higher than mediator reaction ($R_{ES}$) and two orders of magnitude higher than metallic redox process at the anode ($R_{other}$). The absolute values of CTIs for ferricyanide and oxygen based MFCs differ due to the differences between the biocatalyst properties in the MFCs tested. Nevertheless the trend of $R_A$, $R_{ES}$, and $R_{other}$ remain the same for both MFCs. Assuming a two electron transfer for the lactate oxidation and one electron transfer for the mediator reaction, the equilibrium exchange current density ($I_{ex}$) was calculated to be between 1.5 and 2.2 $\mu A/cm^2$ for the substrate oxidation and between 33 and 82 $\mu A/cm^2$ for the mediator reaction. The $I_{ex}$ for lactate oxidation determined in this study is nearly 10 times higher than that of acetate oxidation on a fully grown biofilm composed of a mixed bacterial consortium observed in our earlier study (Ramasamy et al., 2008b). The difference can be attributed to the higher oxidizing potential of lactate ($\Delta G^0 = -518$ kJ/mol) than that of acetate ($\Delta G^0 = -369$ kJ/mol) in the redox potential ladder (Heijnen, 1999; Thauer et al., 1977). The $I_{ex}$ values indicate that the electron transfer ($R_{ES}$) from the mediator to the electrode (or to a surface bound metal, $R_{other}$) is over an order of magnitude faster than the charge transfer from the microbes to the mediator ($R_A$) and hence the latter can be rightfully referred to as the anode rate limiting step under equilibrium conditions. The possible causes for this limitation may include but not limited to: (a) slow diffusion of mediators in and out of the bacterial cell; (b) slower oxidation of the substrate by the bacteria; and (c) high over-potential requirement for the transfer of electrons to the mediators. A series of further microbial and biochemical investigations must be followed to rigorously define these mechanisms. High anode over-potentials in closed circuit MFCs may influence the absolute values of the resistances reported in Table I, but not alter the ratio of $R_A/R_{ES}$ significantly. Equivalent circuit fitting of the data from MFC supplemented with riboflavin (Fig. 2) indicated that upon adding 5 $\mu$M riboflavin, the CTI for substrate oxidation decreased by over 80% from 1,094 to 171 $\Omega$ at open circuit conditions.

Table II reports a similar set of fitted parameters from the cathode EIS data. The $R_{ohm}$ values reported in this table include the contributions from CEM membrane (ferricyanide MFC) or the polycarbonate filter material (oxygen MFC), therefore are higher than anode $R_{ohm}$ values. The values suggest that oxygen reduction on graphite felt possesses 15 times higher CTI than ferricyanide reduction

**Table I.** Parameters from equivalent circuit fitting of the anode EIS data: $R_{ohm}$, ohmic resistance, $R_A$, $R_{ES}$, and $R_{other}$, charge transfer resistances.

| | Lactate/oxygen MFC | | Lactate/ferricyanide MFC | |
|---|---|---|---|---|
| Anode circuit parameters | Values | Influencing factors | Values | Influencing factors |
| $R_{ohm}$ ($\Omega$) | 2 | Electrolyte | 1 | Electrolyte |
| $R_A$ ($\Omega$) | 291 | Substrate oxidation | 417 | Substrate oxidation |
| $R_{ES}$ ($\Omega$) | 39 | Mediator oxidation | 16 | Mediator oxidation |
| $R_{other}$ ($\Omega$) | 4 | Other redox processes | 4 | Other redox processes |

**Table II.** Parameters from equivalent circuit fitting of the cathode EIS data: $R_{ohm}$, ohmic resistance; $R_C$, $R_{ES}$, and $R_{other}$, charge transfer resistances.

| | Lactate/oxygen MFC | | Lactate/ferricyanide MFC | |
|---|---|---|---|---|
| Cathode circuit parameters | Values | Influencing factors | Values | Influencing factors |
| $R_{ohm}$ ($\Omega$) | 12 | Electrolyte + filter | 20 | Electrolyte + membrane |
| $R_C$ ($\Omega$) | 1,554 | Oxygen reduction | 110 | Ferricyanide reduction |
| $R_{ES}$ ($\Omega$) | 77 | Mediator reaction | — | — |
| $R_{other}$ ($\Omega$) | 6 | Other redox processes | 11 | Other redox processes |

consistent with the slow kinetics of oxygen reduction on non-catalyzed surfaces. Interestingly, the exchange current densities for oxygen and ferricyanide reduction on graphite felt at the cathode were 0.2 and 5.8 $\mu A/cm^2$ respectively, which is on the same order of magnitude as the substrate oxidation at the anode under open circuit conditions. The result suggests that at equilibrium conditions, both the anode and cathode charge transfer reactions proceed at comparable rates when a suitable microbe–electron donor pair is used. A variation in this behavior is expected when the MFC is polarized under closed circuit operation using a resistive load, where over-potentials play the dominant role over biochemical kinetics. In closed circuit conditions with no mass transfer limitations, a lower anodic electron transfer rate is likely to limit the MFC performance. It also appears that electro-active species (e.g., metals) in the anolyte diffuse into the cathode chamber through the polycarbonate filter and CEM where they participate in electrochemical reduction process ($R_{other}$). While the $R_{ES}$ and $R_{other}$ values confirm the presence of two distinct reactions at the cathode (Table II), the attribution of these resistances to the mediator and metal reduction processes is consistent with the model system described in Figure 1 and the results shown in Appendix Fig. A1.

The timing and extent of mediator secretion and the physiological role of mediators in respiratory mechanism of *S. oneidensis* are not well understood. While the details of the mediator role in an MFC reactor are in some dispute (vonCanstein et al., 2008; Ward et al., 2004), it is clear that endogenously produced mediators are released from bacterial cells and the presence of those molecules will influence redox processes at the anode surface and within the biofilm. For example, Marsili et al. (2008) observed a 70% reduction in power output when the anode medium (containing presumed mediator) was replaced with a fresh medium. They also found that only 8% of the flavins were adsorbed on the electrode, consistent with the mediators produced by *Shewanella* being dispersed throughout the anolyte, allowing further redox exchanges with the planktonic cells. In complementary experiments, we have found that when supplemented with artificial mediators, *Shewanella* based MFCs attain steady state within 20 h after culture inoculation (unpublished data). The period is insufficient for complete biofilm formation (Ramasamy et al., 2008a). In this system, it appears that a major mode of electron transport must involve mediators, and most likely planktonic bacteria. The experiments and analyses summarized here show applicability of EIS for interpretation of MFC conditions and possibly a means to build insight to soluble mediators' relationships to the physiology of *Shewanella oneidensis* and similar microorganisms.

## Conclusions

Impedance spectroscopy provides a valuable addition to biochemical and spectroscopic techniques for the analysis of biologically synthesized mediators. The technique allows for a non-intrusive on-line detection of secreted redox-active molecules in an active MFC. The soluble mediators released by *Shewanella* yield distinct time constants for electrochemical reactions in the impedance plots. The redox processes involving mediator and electrodes are 10–15 times faster than the bio-electrochemical oxidation of substrate, suggesting that substrate oxidation is the rate limiting step on the anode. The observation provides a basis to examine the effect from addition of various soluble mediators to experimental MFCs. The EIS technique does not provide information about the type or chemical properties of the redox mediator. Neither would it directly provide the redox potential for endogenous mediators. Accordingly, EIS does not displace other analytical methods, but is a complementary approach to define electrochemical contributions of redox active molecules. Future studies of the model MFC should aim to correlate the microbial metabolism, CV, and EIS data in order to gain a deeper understanding on the bioelectrochemistry of extracellular mediators and their implications in MFCs.

This study was funded by the Material Science Directorate of the U.S. Air Force Research Laboratory, and the Air Force Office of Scientific Research (Walt Kozumbo, Program Manager). Venkataramana Gadhamshetty was supported through a post-doctoral fellowship from Oak Ridge Institute for Science and Education.

## Appendix

## EIS Response of an Abiotic Cell

In order to confirm that the high frequency redox response is not related to biological processes or molecules in the MFC, separate EIS measurements were performed on a control reactor that eliminated both substrate and mediator redox contributions to the EIS response. Since the bacteria

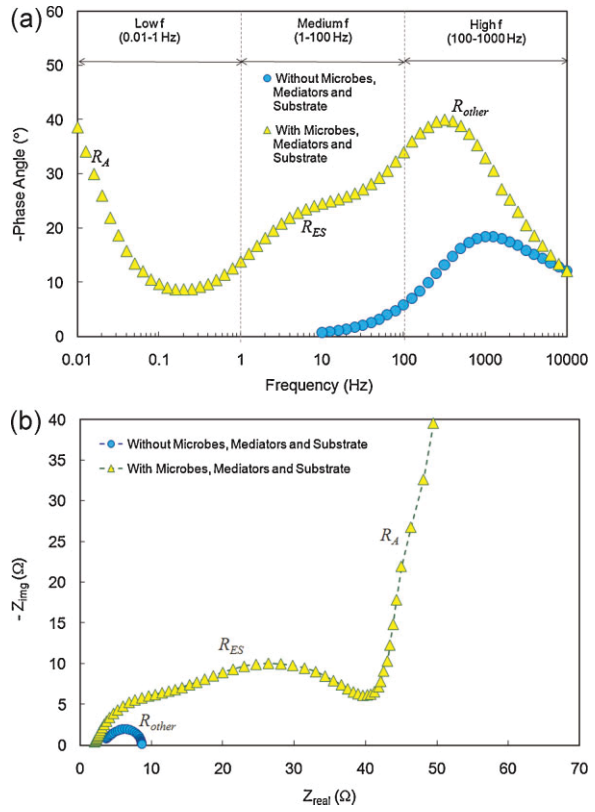


**Figure A1.** Comparison of the EIS response from MFC reactors containing defined media alone (circles) and an active MFC including *S. oneidensis* DSP10 and substrate (triangles) showing the existence of $R_{other}$ for non-biological systems. **a**: Bode phase angle plots and; (**b**) Nyquist plots for the anode. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

may be synthesizing a redox mediator continuously, the control cell was not inoculated (abiotic). The MFC reactor for the control measurement was configured identically to the operating MFCs and contained defined medium, without lactate. The approach eliminates the effects of substrate ($R_A$) and mediators ($R_{ES}$) and thus delineates $R_{other}$ contribution within the defined media. Similar to the model MFCs used in our main study, the control reactor exhibited a charge transfer response between the frequencies 10 kHz and 100 Hz (Fig. A1). The result suggests that the high frequency redox process arises from the electrochemically active species, for example, metal ions, present in the defined media and is not attributable to any putative extracellular mediators. The $R_{other}$ for this reactor was determined to be 4.3 Ω which falls close to the $R_{other}$ values for model operating MFCs (4 Ω) containing *S. oneidensis* DSP10, its associated extracellular mediators, and lactate.

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